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The overall objective of this project is to develop an assay to detect small numbers of breast cancer cells in a patient's peripheral blood. This assay would be used to test the hypothesis that the presence of circulating breast cancer cells would be predictive of stage at presentation or relapse. The first stage of this project has been to optimize a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantify breast cancer-specific RNA molecules in the peripheral blood. The result of this initial work was to select keratin 19 (K19) RNA as the best target for detecting small numbers of breast cancer cells. We have also selected an internal reference RNA ( $\beta_2$ -microglobulin), and we have optimized our protocol for extracting RNA from patient blood samples. The second phase of this project has been to develop methods to enrich blood samples for tumor cells, thereby increasing the sensitivity of the assay. Having optimized these parameters, we plan to use the qRT-PCR assay for K19 RNA to study patients with breast cancer, both at the time of presentation and after treatment.

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## INTRODUCTION

The overall objective of this project is to develop an assay to detect small numbers of breast cancer cells in a patient's peripheral blood. This assay will be used to test the hypothesis that the presence of circulating breast cancer cells is predictive of stage at presentation or relapse. The first stage of this project was to optimize a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantify breast cancer-specific RNA molecules in the peripheral blood. The result of this initial work was to select keratin 19 (K19) RNA as the best target for detecting small numbers of breast cancer cells. We also selected an internal reference RNA ( $\beta_2$ -microglobulin), and we optimized our protocol for extracting RNA from patient blood samples. The second stage of this work has been to develop ways to enrich peripheral blood specimens for tumor cells, thereby increasing the sensitivity of the assay. Having optimized these parameters, we plan to use the qRT-PCR assay for K19 RNA to study patients with breast cancer, both at the time of presentation and after treatment.

## WORK ACCOMPLISHED

### Task 1: To develop molecular tests to quantify breast cancer cells in the peripheral blood

During the past year, we have devoted our efforts to increasing the sensitivity of the qRT-PCR assay to detect small numbers of circulating breast cancer cells. In our last annual report, I indicated that we were able to detect as few as 5 tumor cells in  $10^6$  white cells. This level of sensitivity is based on experiments in which tumor cells grown in culture were mixed with non-tumor cells. The sensitivity of the assay was higher when tumor cell RNA was mixed with water, indicating that the presence of background RNA decreases the sensitivity of the RT-PCR reaction. Thus, if we could reduce the background RNA in the assay, we should improve the assay's sensitivity. This is the rationale for our efforts of the past year, in which we have attempted to enrich samples for tumor cells prior to RNA isolation. Our work was slowed considerably due to a 4-month period in which we were having technical difficulties with the Applied Biosystems 7700 sequence detector used to perform our assays. As a result, we do not have the number of reportable outcomes I would have liked. These problems have now been solved, and we are again making satisfactory progress.

#### *Ficoll-Hypaque Density Gradient Separation*

Ficoll-Hypaque can be used to separate whole blood based on cell density. Red blood cells and granulocytes are denser than standard Ficoll-Hypaque, so these cells can be removed by layering whole blood over a Ficoll-Hypaque cushion, and then performing centrifugation. Our initial experiments using whole blood spiked with SKBR3 cells showed that RNA isolated from mononuclear cells that "float" on Ficoll-Hypaque is amplified by PCR more efficiently than RNA isolated from spiked whole blood. Additional efficiency was obtained when red cells were lysed with hypotonic ammonium chloride prior to Ficoll-Hypaque separation. The increase in assay sensitivity provided by Ficoll separation was one log, or 5 tumor cells in  $10^7$  white cells.

#### *Positive Selection with Magnetic Beads*

Magnetic bead enrichment has been used successfully to selectively purify epithelial cells from blood (1). To test the utility of magnetic beads to enrich for breast cancer cells in blood we used Dynabeads (Dynal Biotech, Oslo, Norway) to purify SKBR3 cells that had been added to whole blood samples. Successful enrichment was verified using flow cytometry. In an experiment in which qRT-PCR was performed in parallel on Ficoll-enriched and Dynabead-enriched samples, the Dynabeads produced a 10-fold increase in sensitivity, although the sensitivity of this experiment was lower than in most experiments. The enrichment for epithelial cells was verified by flow cytometry. Despite the enrichment observed, there was still considerable contamination of the enriched cells by leukocytes as evident from the  $\beta_2$ -microglobulin signal obtained from the enriched

specimens, from the flow cytometry results that show significant numbers of non-epithelial cells, and from microscopic examination of the enriched cell specimens.

#### *Negative Selection with RosetteSep*

Because of the leukocyte contamination observed with Dynabead selection, and because it was possible that the Dynabeads were less than 100% efficient at recovering tumor cells, we explored the possibility of depleting leukocytes rather than enriching for tumor cells. RosetteSep is a product of Stem Cell Technologies (Vancouver, BC, Canada) that uses a mixture of anti-leukocyte antibodies to remove white cells by rosetting and density gradient separation. We compared the sensitivity of the magnetic bead enrichment and RosetteSep negative selection methods on blood samples spiked with SKBR3 cells. Magnetic bead enrichment appeared to result in higher amounts of K19 mRNA in samples spiked with as few as one SKBR3 cell per million white cells. However the RosetteSep method gave a detectable K19 signal with one SKBR3 cell per ten million white cells. We need to explore further which method is optimal for maximizing the sensitivity of the K19 qRT-PCR assay.

#### *Establishment of reference range*

Because we have taken additional steps to increase the sensitivity of the qRT-PCR assay, it will be necessary to establish a new reference range with normal blood donors. Preliminary experiments using either positive or negative selection enrichment methods indicate that K19 mRNA is present at very low levels in the peripheral blood of normal volunteers. Therefore, it will be important to establish the maximum amount of K19 mRNA that is present in normal individuals to determine the significance of finding a given level of K19 mRNA in breast cancer patients. This work is currently under way.

#### **Task 2: To apply the test to untreated breast cancer patients**

Since we have spent a considerable amount of time maximizing the sensitivity of our assay, we have not yet begun collecting specimens from breast cancer patients to test with the optimized methods. We plan to collect specimens from the University of Washington Medical Center (supervised by Dr. Melanie Palomares) and from the Harborview Medical Center (supervised by Dr. Hannah Linden). In addition, we will receive additional patient specimens through collaboration with Dr. Nancy Kiviat. Dr. Kiviat has an ongoing research program in Senegal and has collected specimens from patients with primary diagnoses of breast cancer in Africa. She will be sharing RNA from these samples for our study.

We will be collecting peripheral blood samples from patients with new breast cancer diagnoses with all clinical stages of disease. We would like to get as many samples as possible from patients with Stage III and IV disease, since we expect these to provide more positive results. This will be the primary focus of our work in the coming year.

#### **Task 3: To apply the test to treated breast cancer patients to determine if the test can predict disease progression**

Since we are just beginning to collect specimens from first-time patients, we have not yet begun work on this task.

#### **Key Research Accomplishments**

1. Increased sensitivity of the qRT-PCR assay using positive and negative selection for epithelial cells.
2. Solved formidable technical problems with the instrument used to perform our assays
3. Establishment of working relationship with oncology clinics in Seattle and Senegal from which to get patient specimens

**Reportable Outcomes***Training supported by this award*

Dr. Melanie Palomares, a Senior Fellow in Oncology, conducted this research project in partial fulfillment of her requirements for board eligibility in Hematology-Oncology.

Joyce Addo, an undergraduate at Delaware State University, worked in the laboratory this summer under the direct supervision of Dr. Palomares. She presented a poster of her work to our research group, and will be presenting her poster when she returns to college in Delaware.

**Conclusions**

The work accomplished so far has been to validate an assay to quantitate K19 RNA in the blood of breast cancer patients, which should reflect the number of circulating breast cancer cells. Although we have not yet begun clinical trials with this assay, we have demonstrated that we should be able to detect 1 breast cancer cell in  $10^7$  white blood cells. In the next phase of our study, we will determine whether this assay has clinical utility for both determining a patient's prognosis at initial presentation and predicting the likelihood of progressive disease on follow-up. We will also be evaluating whether using magnetic bead enrichment of epithelial cells or depletion of leukocytes will improve the assay sensitivity. If our efforts are successful, this assay may come to be used in the routine care of cancer patients. We hope that by detecting cancer cells in the blood, patients may be treated earlier for recurrent/metastatic disease and that this will translate into improved patient survival.

**Reference**

1. Martin, V. M., Siewert, C., Scharl, A., Harms, T., Heinze, R., Öhl, S., Radbruch, A., Miltenyi, S. and Schmitz, J. (1998) Immunomagnetic enrichment of disseminated epithelial tumor cells from peripheral blood by MACS. *Exp. Hematol.*, **26**, 252-264.